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ANTIOXIDANT ACTIVITY *IN VITRO* AND HEPATOPROTECTIVE EFFECT OF *PHLOMIS MAXIMOWICZII IN VIVO*

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Abstract

Background: A number of medicinal plants and there compounds played a major role in the treatment of hepatic disorders. They were widely used for the treatment of these disorders, and oxidant stress injury was one of the liver injury mechanisms. The present study evaluated the antioxidant activity and the hepatoprotective effect of each extracts of *Phlomis maximowiczii*.

Materials and Methods: The antioxidant activity was assayed by the methods of ABTS, FRAP and DPPH *in vitro*. Hepatoprotective effect of *P. maximowiczii* extracts was examined using carbon tetrachloride-induced acute liver injury in mice.

Results: *P. maximowiczii n*-butanol (PMBU) extract, ABTS (IC_{50}=18.96 μg/mL), DPPH (IC_{50}=25.15 μg/mL), and FRAP ($RACT_{50}$=2775.6±144.18 μmol/g), showed higher scavenging capacity than that of *P. maximowiczii* ethyl acetate (PMEA). The *n*-butanol extract could significantly reduce the level of GPT, GOT and MDA (P<0.05, P<0.001 and P<0.001, respectively) and increase the level of SOD (P<0.001), respectively.

Conclusion: The antioxidant activity of *n*-butanol extract *in vitro* was related with the level of MDA and SOD *in vivo*, and hepatoprotective effect of *n*-butanol extract also had relationship with its antioxidant activity *in vivo*.

Key words: *Phlomis maximowiczii*, anti-oxidation, acute liver in jury, carbon tetrachloride.

Introduction

As a metabolic organ, the liver is vulnerable to injury by a variety of xenobiotics, such as CCl_4, ethanol and acetaminophen which are metabolized by cytochrome P450 2E1 (CYP 2E1), (Sun et al., 2001). Carbon tetrachloride (CCl_4), as a well-known hepatotoxin used as chemical inducer of experimental liver injury in a range of laboratory animals (Recknagel et al., 1989). The mechanism of CCl_4-induced acute liver injury is widely accepted as metabolized to a highly reactive trichloromethyl radical ($CCl_3^{\cdot}$), by cytochrome P450 in liver. $CCl_3^{\cdot}$ in liver, and can leads to lipid peroxidation, as well as to hepatocellular membrane damage (Ohta et al., 1998; Drill, 1952). Natural antioxidants have been accepted widely as preventing the deleterious effects of toxic agents by scavenging free radicals and other reactive oxygen species (Domitrović et al., 2011).

Phlomis maximowiczii, belongs to Labiatae family, is widely distributed in Asia. It has been used in various folk medications for the treatment of inflammatory cold, haemorrhage, and fever (Xie et al., 1996). Fat-soluble ingredients of *P. maximowiczii* in our previous studies were reported (Gu and Chen, et al., 2012). There is no research of *P. maximowiczii* on chemical research, hepatoprotective and antioxidant effects. In order to investigate the hepatoprotective and antioxidant effects of *P. maximowiczii*, *P. maximowiczii* extracts were assayed using the CCl_4-induced liver injury mice *in vivo*, and the methods of ABTS, FRAP and DPPH *in vitro*.

Materials and Methods

Plant material

Phlomis maximowiczii (Voucher No: 20090723) whole plant was collected in July 2009, from Tianchi Mountain in Henan Province and identified by Professor Changqin Li (Institute of Natural Products, Henan University). Voucher specimen was deposited at the Institute of Natural Products, Henan University, Kaifeng, China.

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Extraction

Dried whole plants of about (2.0 kg), was extracted three times with methanol at room temperature for three days. The total methanol extract was filtered and concentrated *in-vacuo*, and extracted with petroleum ether, ethyl acetate and *n*-butanol respectively. Solution was concentrated under reduced pressure to yield petroleum ether of *P. maximowiczii* (PMPE), ethyl acetate of *P. maximowiczii* (PMEA), and *n*-butanol extracts (PMBU), respectively.

Assays for antioxidant properties of extracts *in vitro*

Scavenging of 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH)

0.1 mL different extracts of *P. maximowiczii* in methanol had been mixed with 3.5 mL 2,2-Diphenyl-1-Picrylhydrazyl (DPPH; Chemical Industry Co. Ltd., Japan), methanol solution (0.06 mmol/L). The solution was measured at 515 nm after 30 min at room temperature with PG, BHA and BHT as positive control (Kang et al., 2010). The antioxidant activity was expressed as an IC_{50} value, that is, the concentration in g/mL that inhibits DPPH absorption by 50%, and was calculated from the concentration-effect linear regression curve. The radical scavenging activity (RSA), of extracts was expressed in terms of percentage inhibition of DPPH radical by extracts and was calculated as follows:

RSA (DPPH. Inhibition, %) = $[(A_B\text{-}A_T)/A_B] \times 100$

Where, A_B = Absorbance of radical blank (DPPH. without extracts)

A_T = Absorbance of test sample (DPPH. with extracts)

Scavenging of 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)(ABTS)

The different extracts of *P. maximowiczii* (0.15 mL) were mixed with ABTS (Fluka; USA), radical stock solution (2.85 mL), and incubated at 37 °C. The absorbance was observed at 734 nm after 10 min with PG, BHA and BHT as positive control (Kang and Wang et al., 2010). The percentage inhibition of $ABTS^{\cdot+}$was calculated using the formula: % Inhibition = $[(A_0\text{-}A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample and the standard compound.

Ferric reducing antioxidant power (FRAP) reducing activity assay

The *P. maximowiczii* (0.2 mL), and fresh prepared TPTZ (Acros organics; USA) stock solution (3.8 mL) were mixed and incubated at 37°C for 30 min. The absorbance was measured at 593 nm (Thipong et al., 2006). Trolox (Aldrich; USA), was used as a reference standard. The standard curve was linear between 25 and 400 mol/L Trolox (R=0.999). Results were expressed in µmol Trolox equivalents (TEAC), (TE)/g sample. In this study, $RACT_{50}$ was used to express Trolox equivalent ($RACT_{50}$= the concentration of Trolox cleared 50% free radical/ the concentration of compound or condensate cleared 50% free radical).

Hepatoprotective effect of *P. Maximowiczii in vivo*

Materials and animals in experiments *in vivo*

Male KM normal rats weighting 20±2 g were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, Hennan, China), (12 h light/dark cycle, 25°C and humidity 45 to 65%), and were fed with standard rodent diet and water *ad libitum*. All animal procedures were approved by the ethical committee in accordance with Institute ethical committee guidelines' for Animal Experimentation and Care. Animals were housed in polycarbonate cages.

The materials include maleicdialdehyde (MDA, No: 20120724), superoxide dismutase (SOD, No: 20111201), glutamate-pyruvate transaminase (GPT, No: 20120717) and glutamate-oxaloacetate transaminase (GOT, No: 20120720) from the Nanjing Jianchen Bioengineering Institute (Jiangsu, China). Coomassie brilliant blue G250 from the Shanghai Chemical Reagent Company (Shanghai, China, No: 20070867). CCl_4 were purchased from Sigma Chemical Co. Bifendate pills (No: 10031) were purchased from pharmaceutical Co. Ltd., Zhejiang, China. Bovine serum albumin from Beijing AoBoxing Research Bio-Tech co., Ltd (Beijing, China).

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Experimental design and treatment schedule

Mice were randomly divided into nine groups with 10 mice in each group; and normal control group, CCl_4 model group, bifendate (75 mg/kg), group, PMBU (600, 300 and 150 mg/kg, respectively), groups and the PMEA (800, 400 and 200 mg/kg, respectively), groups. Mice were administered orally by gastric gavage with different doses of PMBU, PMBU and bifendate at a volume of 10 mL/kg once a day for 8 days. The normal control group and the CCl_4 model group were administered with an equivalent volume of distilled water. On the eighth day, at 2 h after the final administration, except of normal control group, the mice in other groups were intra-peritoneally injected with CCl_4 diluted in olive oil at the dose of 0.05 mL/kg body weight, and the normal control group was injected with an equivalent volume of olive oil alone (Chen et al., 2004).

At 16 hr after the CCl_4 injection, each mouse was weighed and then killed under light ether anesthesia for blood collection via puncture of the retro-orbital venous plexus. Serum was obtained from the collected blood by centrifugation immediately after death. Liver homogenate was homogenized with physiological saline. The homogenates were centrifuged at 3000 rpm for 10 min at 4°C and clear supernatants were used immediately for assessment of MDA and SOD (Gong et al., 2012).

Biochemical analyses (Lowry et al., 1951)

The protein content in homogenates was assayed by the method of using bovine plasma albumin as a standard. The levels of GOT, GPT, SOD and MDA were measured following the commercial kit's instructions.

Statistical analysis

Statistical analyses were carried out using SPSS 17.0 software. The overall significance of the results was examined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $P<0.05$ was considered statistically significant. All values were expressed as mean values ± standard deviation (SD).

Results

Assay for free radical scavenging activity *in vitro*

The antioxidant activity of *P. maximowiczii* with half inhibitory concentration (IC_{50}), and Trolox equivalent ($RACT_{50}$), is shown in Table 1. In ABTS assay, the antioxidant activity of PMBU (IC_{50}=18.96 μg/mL) was higher than that of PMEA (IC_{50} = 36.73 μg/mL). In DPPH assay, the antioxidant activity of PMBU (IC_{50}=25.15 μg/mL) was lower than that of BHT (IC_{50} = 18.71 μg/mL). In FRAP assay, the antioxidant activity of PMBU ($RACT_{50}$ = 2775.6±144.18 μmol/g) was higher than that of PG ($RACT_{50}$ = 1581.68±97.41 μmol/g). The results showed that the antioxidant activity of PMBU had the highest antioxidant activity *in vitro*.

Table 1 Antioxidation *in vitro* of *Phlomis maximowiczii*

Sample	ABTS radical scavenging capacity IC_{50} (μg/mL)	DPPH radical scavenging capacity IC_{50} (μg/mL)	Ferric reducing antioxidant power $RACT_{50}$(μmol/g)
PMPE	NT	NT	502.4±9.88
PMEA	36.73	NT	606.4±5.64
PMBU	18.96	25.15	2775.6±144.18
PG	0.81	0.89	1581.68±97.41
BHA	1.95	3.2	NT
BHT	7.72	18.71	NT

NT unavailable, because of low activity. BHT, BHA and PG were used as positive control.

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Effect of PMBU and PMEA on GPT and GOT in serum

The level of GPT and GOT in normal and acute liver injury mice is shown in Table 2. The level of hepatic GPT and GOT in CCl_4-treated mice increased significantly compared with the normal control (P<0.001), and indicated that the acute liver injury mice model was established. Compared with model group, the level of GPT and GOT of PMBU group decreased significantly (P<0.05 and P<0.001, respectively), showed that had a therapeutic effect. Compared with bifendate (75 mg/kg), as positive control, intra-gastric administration of PMBU had no significantly difference (P>0.05) and presented dose-dependent manner. (Table 2 and figure 1).

Effect of PMBU and PMEA on MDA and SOD in liver

The effects of different doses of PMBU and PMEA on the level of SOD, MDA in normal and CCl_4-induced liver injury mice are shown in Table 3. The level of MDA in liver significantly increased in liver injury control mice (P<0.001), and the level of SOD in liver significantly decreased (P<0.001), when compared with normal control group. The level of MDA in liver only in administration of PMEA (400 mg/kg), had no significant reduction (P>0.05), the other treatment groups had significantly reduced (P<0.001). The level of SOD of each treatment groups had significant increase (P<0.001 and P<0.01, respectively), when compared with model group. Compared with bifendate (75 mg/kg), intra-gastric administration of PMBU showed no significant difference (P>0.05) and presented dose-dependent manner. (Table 3 and Figure 2 and 3).

Table 2 Effect of *Phlomis maximowiczii* on the level of GOT and GPT in acute liver injury in mice

Group	Dose (mg/kg)	GPT (IU/L)	GOT (IU/L)
Model control	/	$1075.95\pm5.40^{\Delta\Delta\Delta}$	$635.89\pm51.91^{\Delta\Delta\Delta}$
Bifendate	75	$577.31\pm80.36^{***}$	$283.51\pm60.99^{***}$
normal control	/	$43.40\pm6.62^{***}$	$130.74\pm6.55^{***}$
PMEA	800	1040.22±75.23	$260.27\pm53.76^{***}$
PMEA	400	1951.00±465.77	627.42±80.04
PMEA	200	$529.66\pm17.23^{***}$	$287.05\pm17.73^{***}$
PMBU	600	$805.46\pm32.44^{*}$	$218.12\pm53.27^{***}$
PMBU	300	$813.32\pm32.61^{*}$	$371.57\pm19.23^{***}$
PMBU	150	$698.06\pm79.58^{*}$	$485.22\pm60.79^{**}$

Data expressed as means ± SD (n=10). Bifendate was as the positive control drug. P<0.05, $^{\Delta\Delta}P$<0.01 and $^{\Delta\Delta\Delta}P$<0.001, normal group compared with CCl_4-induced acute liver injury. $^{*}P$<0.05, $^{**}P$<0.01, and $^{***}P$<0.001, treated group compared with CCl_4-induced acute liver injury.

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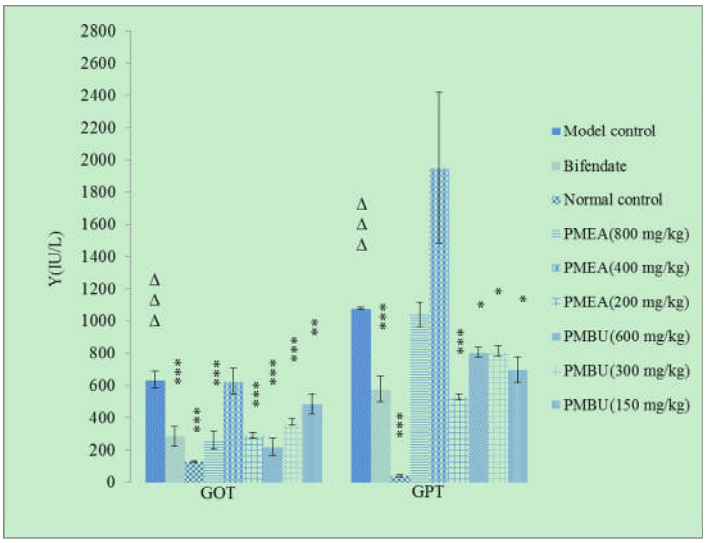

Figure 1: Effect of PMBU and PMEA on GPT and GOT in serum

Table 3: Effect of *Phlomis maximowiczii* on the level of SOD and MDA in acute liver injury in mice

Group	Dose (mg/kg)	SOD (U/mL)	MDA (nmol/mL)
Model control	/	$436.81\pm29.72^{\Delta\Delta\Delta}$	$14.73\pm2.10^{\Delta\Delta\Delta}$
Bifendate	75	$741.03\pm38.00^{***}$	$9.60\pm1.95^{***}$
Normal control	/	$732.27\pm25.16^{***}$	$9.88\pm1.44^{***}$
PMEA	800	$571.91\pm19.88^{***}$	$10.60\pm0.26^{***}$
PMEA	400	$542.144\pm28.85^{***}$	12.88 ± 0.89
PMEA	200	$378.44\pm43.21^{*}$	$7.19\pm0.62^{***}$
PMBU	600	$741.65\pm48.41^{***}$	$9.67\pm1.15^{***}$
PMBU	300	$635.92\pm19.92^{***}$	$8.47\pm0.90^{***}$
PMBU	150	$492.43\pm16.97^{*}$	$8.93\pm1.67^{***}$

Data expressed as means ± SD (n=10). Bifendate was as the positive control drug. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ and $^{\Delta\Delta\Delta}P<0.001$, normal group compared with CCl_4-induced acute liver injury. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, treated group compared with CCl_4-induced acute liver injury.

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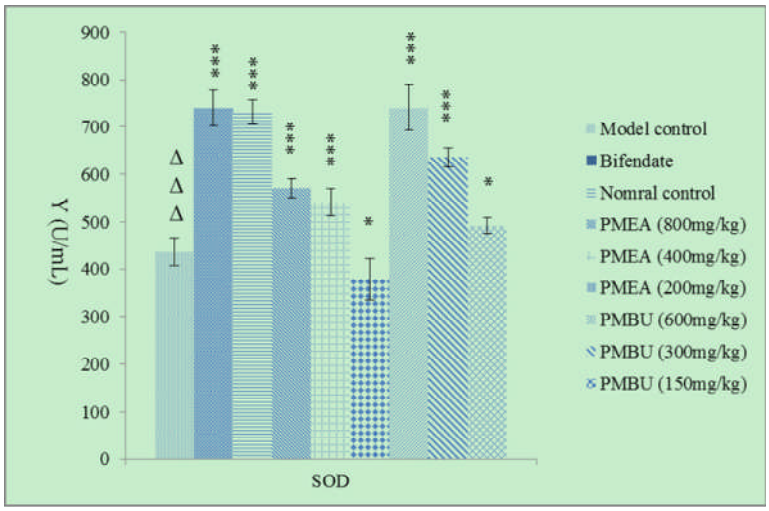

Figure 2: Effect of *Phlomis maximowiczii* on the level of SOD in acute liver injury in mice.

Discussion

Acute and chronic liver diseases is mostly induced by viral hepatitis, alcoholism, iron overload or drug toxicity. Among these types of liver injuries, there is consistent evidence of enhanced production of free radicals and/or a significant decrease in antioxidant defense mechanisms (Hoek and Pastorino, 2002). Carbon tetrachloride (CCl_4) is a well-established model for screening hepato-protective drugs, with a marked elevation in the serum levels of the aminotransferases enzymes GOT and GPT. Hepatic cells damaged by free radicals and released these enzymes into the blood. Results showed that a significant in the level of GOT and GPT in CCl_4-treated mice (P<0.001). Levels of GPT and GOT, MDA decreased significantly (P<0.05 and P<0.01 and P<0.001, respectively), and SOD in administration of each dose group of PMBU. Results of scavenging activity of *P. maximowiczii* showed that *n*-butanol extract showed higher antioxidant activity than that of ethyl acetate extracts *in vitro*.

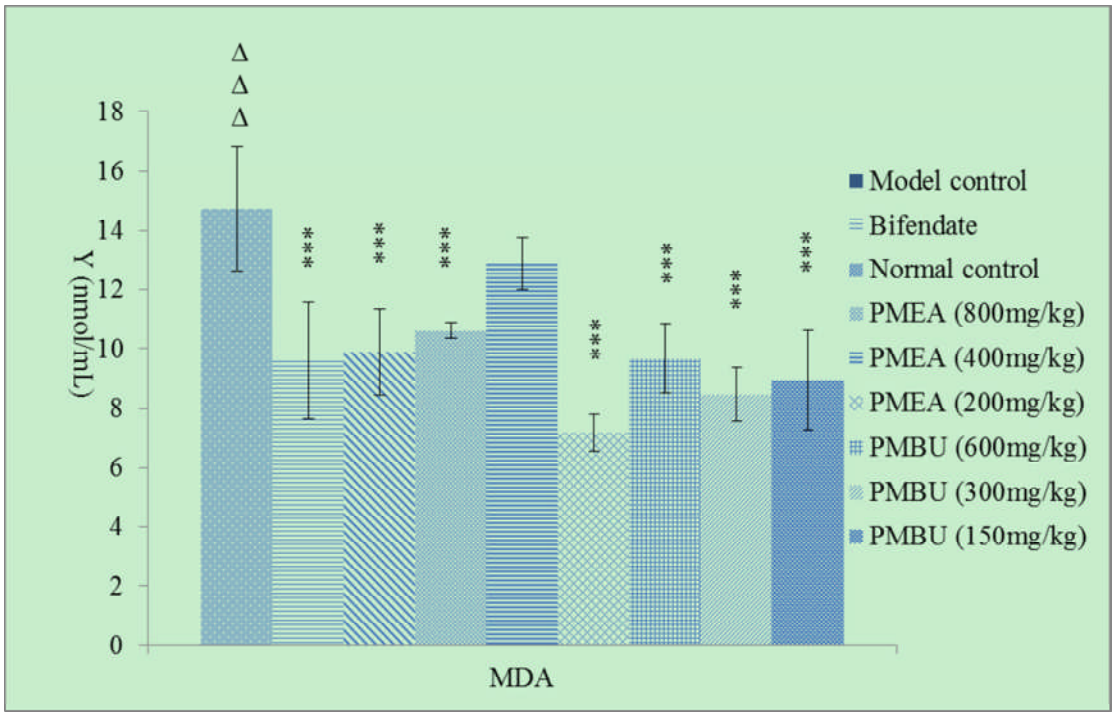

Figure 3: Effect of *Phlomis maximowiczii* on the level of MDA in acute liver injury in mice

Antioxidant is one of the hepatoprotective mechanisms to decrease lipid peroxidation and oxidant stress. Therefore, protective of PMBU for CCl_4-induced liver injury in mice was better than that of PMEA. The result indicates that PMBU had very good hepatoprotective activity. Further

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work is necessary to isolate active ingredients and elucidate the actual mechanism involved in the hepatoprotective and antioxidant activity of this plant.

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